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BRIEF REPORT

Characterization of the full-length genomic sequences of vesicular stomatitis Cocal and Alagoas viruses

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Abstract In Brazil and Argentina, vesicular stomatitis (VS) is caused by distinct viral strains serologically related to the classical vesicular stomatitis virus Indiana (VSIV), namely VS Indiana-2 (VSIV-2) and VS Indiana-3 (VSIV-3). Here we describe the full-length genomic sequences and organization of the prototype strains of VSIV-2 Cocal virus (COCV) and VSIV-3 Alagoas virus (VSAV). These viruses showed similar genomic organizations to VSIV field isolates except that the non-structural C′/C proteins, markedly conserved throughout the vesiculoviruses, were absent in VSAV. Phylogenetic analyses consistently grouped COCV, VSAV and VSIV in a monophyletic group distinct from VSNJV, supporting the classification of these viruses within the Indiana serogroup.

VS affects cattle, horses and pigs in North, Central and South America and causes serious economic losses [17]. Its clinical presentation is similar to foot-and-mouth disease, making differential diagnosis of critical importance. The etiologic agents of VS are members of the family *Rhabdoviridae*, genus *Vesiculovirus* that have been serologically classified into two major serotypes: New Jersey (VSNJV) and Indiana (VSIV). The Indiana serotype has been further divided into three separate serological groups associated with vesicular disease [6]. Indiana type 1 represents the classical Indiana strains (VSIV). Cocal virus (COCV) is the prototype virus of the Indiana 2 subtype and was originally isolated from mites collected from rice rats in Trinidad in 1961 and again near Para, Brazil in 1962 [8]. Alagoas virus (VSAV) is the prototype virus of the Indiana 3 subtype and was first isolated from a mule in Alagoas, Brazil, in 1964 [3].

VSNJV and VSIV outbreaks occur in northwest South America (Bolivia, Colombia, Ecuador, Peru, Venezuela), throughout Central America and in North America (Mexico and United States) [10]. In Brazil and Argentina, however, VSNJV and VSIV are not reported, but outbreaks of VS are caused by viruses related to VSIV that have been serologically classified either as VSIV-2 or VSIV-3 [2, 6]. The first VSIV-2 isolate associated with clinical disease was isolated from horses in Salto, Argentina in 1963 and again in Maipu, Argentina, in 1986. VSIV-2 has also been isolated from horses in Rancharia (1966) and Riberao (1979), Brazil [1, 2]. More recently, in 1998, a large outbreak of VSIV-2 occurred in cattle in the states of Santa Catarina and Parana in southern Brazil (Situation of Foot-and-Mouth Disease Eradication Programs in South America in 2002, PANAFTOSA, March 2003 http://www.panaftosa.org.br).

VSIV-3 has caused sporadic outbreaks in cattle in northeast Brazil (Espinosa (1977), Sergipe (1984), and Agulhas Negras (1986)) [1]. While VSIV-3 has never been reported outside of Brazil, five viral isolates collected from naturally infected phlebotomine sand flies in Colombia in 1986 were classified serologically as VSIV-3. Although antibodies to these viruses were found among the local livestock population, curiously, these viruses were not associated with clinical disease [21].

Previous studies have described the molecular characterization and epidemiology of VS strains occurring in

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Fig. 1 Alignment of the 3′ Le (**a**) and the positive-sense Tr (TrC) regions (**b**) of VSIV, COCV and VSAV. Positions of sequence identity are indicated by *dashes*. The regions necessary for VSIV mRNA transcription initiation are shown *boxed*. Conserved areas of terminal complementation, hence sequence identity between the Le and TrC, are shown in *grey*

Central and North America [12, 14, 15, 18]. However, there is little information on the molecular characteristics of viruses from Brazil and Argentina, with only a few partial genomic sequences of COCV and VSAV available in genomic databases. Here we report the characterization of the full-length genomic sequences of COCV and VSAV, the prototypes of viruses causing vesicular stomatitis in livestock in Brazil and Argentina.

Reference strains of COCV and VSAV were obtained from the USDA-APHIS Foreign Animal Disease Diagnostic Laboratory (FADDL). Virus nomenclature follows the VIIIth Report of the International Committee on Taxonomy of Viruses (ICTV) [5]. Viruses were passed once by infecting BHK-21 cells at a multiplicity of infection of 0.1 or less. Except for the termini, genomic sequences were obtained and analyzed as previously described [16]. To sequence the genomic termini, RNA was extracted from sucrose-gradient-purified virions after an additional passage in BHK-21 cells as described before [18], and a modified 5′/3′ RACE was performed. The 5′ end was sequenced using the 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen) as per the manufacturer's instructions. A poly(A) tail was added to the virion 3′ end using poly(A) polymerase (USB, Cleveland, OH, USA), and the 3′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen) was used to sequence the 3′ end. Primers for PCR and sequencing reactions were designed based on the published sequences of VSIV or based on newly obtained sequences (primer sequences are available from corresponding author upon request).

Phylogenetic analysis was performed using the distance optimality criterion as implemented in PAUP* 4.0b10 [20]. The neighbor-joining trees were bootstrapped 2,000 times to obtain a 95% confidence value. Additional sequences used in our analysis included VSIV-85CLB (Accession AF473865); VSNJV Ogden (Accessions K02379 [N gene], M31873 [P gene], M14553 [M gene], M21416 [G gene] and M29788 [L gene]); Chandipura virus (accessions AY614724 [N and P genes], AF 128868 [M gene], J04350 [G gene] and AJ810083 [L gene]; Isfahan virus (Accession AJ810084) and spring viremia of carp virus (Accession U18101).

The basic genomic structures of COCV and VSAV were similar to that of other vesicular stomatitis viruses, with a 47-nt 3′ leader (Le) sequence followed by the 5 structural genes and a 57-nt (COCV) or 58-nt (VSAV) 5′ trailer (Tr) sequence. Total genomic lengths were 11003 nt and 11070 nt for COCV and VSAV, respectively, which is shorter than the 11155–11336-nt length observed in field isolates of VSIV [18]. Most differences in length were in non-coding regions, particularly in the M and G mRNAs (data not shown). Despite the variability in lengths of the five mRNAs, four of the five predicted structural proteins (N, M, G and L) were within one aa residue in length of each other. The complete genome sequences of COCV and VSAV were submitted to GenBank and assigned accession numbers EU373657 and EU373658, respectively.

Amino Acid Divergene

N	COCV	VSAV	NJ
VSIV	16.1	14.9	31.0
COCV	-	14.7	30.6
VSAV	-	-	31.0

P	COCV	VSAV	NJ
VSIV	40.9	45.4	69.4
COCV	-	40.8	68.2
VSAV	-	-	68.5

M	COCV	VSAV	NJ
VSIV	24.5	24.9	40.2
COCV	-	18.8	41.9
VSAV	-	-	41.5

G	COCV	VSAV	NJ
VSIV	28.0	38.0	51.7
COCV	-	33.5	53.3
VSAV	-	-	53.2

L	COCV	VSAV	NJ
VSIV	23.2	24.2	34.0
COCV	-	21.8	34.8
VSAV	-	-	33.3

Nucleotide Divergence

N	COCV	VSAV	NJ
VSIV	30.1	30.7	37.7
COCV	-	29.6	39.5
VSAV	-	-	40.4

P	COCV	VSAV	NJ
VSIV	47.2	52.9	66.9
COCV	-	44.7	63.8
VSAV	-	-	65.9

M	COCV	VSAV	NJ
VSIV	31.9	35.2	45.9
COCV	-	30.1	47.0
VSAV	-	-	46.5

G	COCV	VSAV	NJ
VSIV	37.1	43.3	53.8
COCV	-	40.8	56.3
VSAV	-	-	53.9

L	COCV	VSAV	NJ
VSIV	36.9	37.7	42.7
COCV	-	35.4	43.4
VSAV	-	-	43.0

Fig. 2 Nucleotide and deduced amino acid divergence scores for the five structural proteins of VSIV, COCV, VSAV and VSNJV as calculated using MEGALIGN (DNASTAR®)

The 3′Le of vesiculoviruses is believed to function as a promoter for transcription of viral mRNAs and viral replication. The 5′ Tr, when in the positive-sense antigenomic state (TrC), is believed to function as a promoter for replication of the negative-sense progeny genomes [23]. The 15 most terminal nucleotides of Le and TrC have been shown to be required for replication of the positive- and negative-sense viral genome, respectively. These nucleotides were completely conserved in the Le of VSIV, COCV and VSAV and at 12 of the 15 sites in the TrC (Fig. 1). As both Le and TrC are thought to have similar promoter functions, it is not surprising that they have high sequence identity, thus terminal complementarity, in both genomic and anti-genomic senses. Previous studies have shown that increased complementarity between the termini enhances the levels of viral replication [22]. The termini of COCV have been reported previously [7, 13] but this is the first report of the termini of VSAV.

Based on divergence scores, N was the most conserved structural protein at both the amino acid and nucleotide levels, followed by M/L, then G, and P was the least conserved (Fig. 2). A great deal of work has been done on functional domains within the different structural proteins of VSIV, and due to their close genetic relationships (Figs. 2, 3), it is not surprising that there are no significant differences in COCV and VSAV at these critical areas. There were four nucleotide differences between our COCV G sequence and one previously deposited in GenBank (AF045556), all outside of the predicted ORF. The only submission in Genbank (AY335184) attributed to VSAV is a spike glycoprotein that is nonfunctional due to mutation. This 259-nt sequence showed no similarity to the VSAV glycoprotein sequence determined in this study or to any other part of the VSAV genome.

The only prominent differences found among the protein coding regions were within the highly variable P

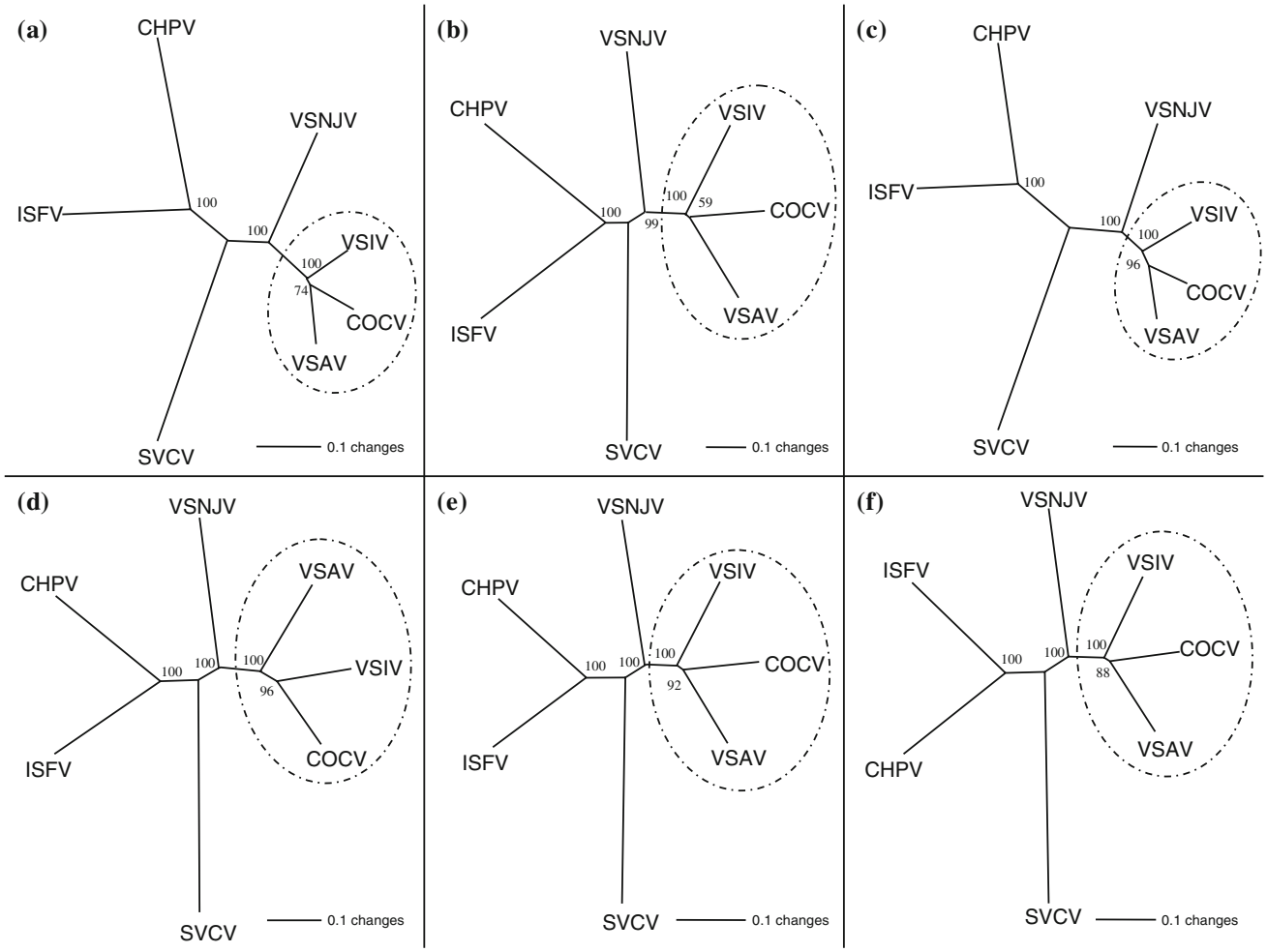

Fig. 3 Phylogenetic relationship of COCV and VSAV with other vesiculoviruses. Bootstrap values >50 calculated with 2,000 replications are indicated at appropriate nodes. The trees were constructed from deduced amino acid alignments based on the N (**a**), P (**b**), M (**c**), G (**d**), L (**e**) or a combined data set of all protein coding regions (**f**). The monophyletic group of VSIV, COCV and VSAV is *circled* (*broken line*) on each tree

gene. Unlike the other four structural proteins, the P gene sequences were more divergent at the protein level (vs. nt level) between VSNJV and the Indiana subtypes (Fig. 2). Hence, the P gene appears to be under different evolutionary constraints than the other four structural proteins. This could be due to the presence of the hypervariable hinge region, which appears to function as a sequence-independent conformational spacer of P, or the presence of the second ORF coding for the C′/C proteins [4].

The C′/C proteins are two small, highly basic, non-structural proteins encoded in a second ORF within the P gene of VSIV [9] and VSNJV [19]. COCV showed an ORF in the second frame of the P gene that could encode multiple proteins between 93 and 15 aa, all predicted to be highly basic. Surprisingly, the C′/C proteins were absent in VSAV. There were no ORFs in the second frame of the P gene that could encode a protein >17 aa residues. Although no specific function has been attributed to them and recombinant viruses lacking these proteins display similar in vitro growth kinetics to wild-type virus [9], these proteins are present in all vesicular stomatitis viruses sequenced to date. Their unexpected absence might represent an adaptation of VSAV to a different vector or other unidentified environmental factor. It would be very interesting to determine the presence/absence of C′/C in field isolates of VSIV-3.

The phylogenetic relationship of COCV and VSAV with other vesiculoviruses was determined based on the deduced amino acid sequence, either individually for each of the five structural proteins or as a combined sequence comprising all deduced protein sequences as they appear genomically (N, P, M, G then L) (Fig. 3). VSIV, COCV and VSAV formed a monophyletic group in all trees, supporting the fact that COCV and VSAV belong within the Indiana serogroup. The only change in tree topology was observed in the tree constructed from the glycoprotein alignment, in which VSAV instead of VSIV was placed closest to the ancestral root of the Indiana serogroup. This observation is difficult to explain, but previous work has shown that different evolutionary histories can be predicted amongst the vesiculoviruses, depending on the gene used in the analysis [11].

The purpose of this work was to characterize the full-length sequences of the prototype viruses causing vesicular stomatitis in livestock in Brazil and Argentina. These viruses have been previously classified serologically as either Indiana-2 (VSIV-2) or Cocal-like and Indiana-3 (VSIV-3) or Alagoas-like viruses. Our analysis of their full-length genomes supports their classification as a subgroup of the Indiana serogroup. A better understanding of these viruses should help in the development of better diagnostic, prevention and control tools for limiting the economic damage that these viruses can inflict on animal health and production.

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